Global responses of *Escherichia coli* to adverse conditions determined by microarrays and FT-IR spectroscopy

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Abstract: The global gene expression and biomolecular composition in an *Escherichia coli* model strain exposed to 10 adverse conditions (sodium chloride, ethanol, glycerol, hydrochloric and acetic acid, sodium hydroxide, heat (46 °C), and cold (15 °C), as well as ethidium bromide and the disinfectant benzalkonium chloride) were determined using DNA microarrays and Fourier transform infrared (FT-IR) spectroscopy. In total, approximately 40% of all investigated genes (1682/4279 genes) significantly changed expression, compared with a nonstressed control. There were, however, only 3 genes (*ygaW* (unknown function), *rmf* (encoding a ribosomal modification factor), and *ghrA* (encoding a glyoxylate/hydroxypyruvate reductase)) that significantly changed expression under all conditions (not including benzalkonium chloride). The FT-IR analysis showed an increase in unsaturated fatty acids during ethanol and cold exposure, and a decrease during acid and heat exposure. Cold conditions induced changes in the carbohydrate composition of the cell, possibly related to the upregulation of outer membrane genes (*glgAP* and *rcsA*). Although some covariance was observed between the 2 data sets, principle component analysis and regression analyses revealed that the gene expression and the biomolecular responses are not well correlated in stressed populations of *E. coli*, underlining the importance of multiple strategies to begin to understand the effect on the whole cell.

Key words: Escherichia coli, global responses, microarray, FT-IR spectroscopy, multivariate data analysis.

Résumé : L'expression génique globale et la composition biomoléculaire d'une souche modèle d'*Escherichia coli* soumise à 10 conditions défavorables (le chlorure de sodium, l'éthanol, le glycérol, l'acide chlorhydrique, l'acide acétique, l'hydroxyde de sodium, la chaleur (46 °C) et le froid (15 °C) ainsi que le bromure d'éthidium et le chlorure de benzalkonium, un désinfectant) ont été déterminées sur des puces à ADN et par spectroscopie infrarouge à transformée de Fourier (IRTF). Au total, environ 40 % de tous les gènes examinés (1682/4279) changeaient de niveau d'expression de façon significative comparativement au contrôle non stressé. Il n'y avait cependant que 3 gènes (*ygaW* (fonction inconnue), *rmf* (codant un facteur de modification ribosomal) et *ghrA* (codant une glyoxylate/hydroxypyruvate réductase)) dont le niveau d'expression changeait significativement sous toutes les conditions (sauf le chlorure de benzalkonium). L'analyse en IRTF a montré une augmentation du contenu en acides gras insaturés lors de l'exposition à l'éthanol et au froid, et une diminution lors d'une exposition aux acides et à la chaleur. Le induisait des changements dans la composition en sucres de la cellule, possiblement en lien avec l'augmentation de l'expression de gènes codant des composantes membranaires (*glgAP* et *rcsA*). Même si une certaine covariance était observée entre les 2 séries de données, les analyses en composantes principales et de régression ont révélé que l'expression génique et la réponse biomoléculaire n'étaient pas en corrélation chez les populations de *E. coli* stressées, et mettent en évidence l'importance d'adopter de multiples stratégies pour comprendre l'effet du stress sur la cellule entière.

Mots-clés : Escherichia coli, réponse globale, puce d'ADN, spectroscopie IRTF, analyse multivariée de données.

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Introduction

Escherichia coli is a widespread bacterium that can survive and adapt to growth under a wide range of conditions. Adaptations are essential to a bimodal lifestyle, either in the primary habitat within animal hosts or in secondary habitats as free living cells in the natural environment (Gordon et al. 2002). It is also the most thoroughly studied bacterium, and is often a preferred model organism, as it uses mainstream metabolic pathways that are similar to the corresponding metabolic functions in other bacteria (Liang et al. 2002). A basic knowledge of how different adverse conditions affect *E. coli* is important when exploring the response potential and understanding how bacteria adapt. There are numerous reports on stress response in *E. coli* (for an overview see Chung et al. (2006) and Storz and Hengge-Aronis (2000)) and the mechanisms behind general stress responses, and more specific responses have been studied in detail. DNA microarray analysis is well known, and today it is a standardized screening method to study global gene expression. The global gene expression of *E. coli* exposed to acetate, glycerol, sodium chloride, and cold and heat stress has previously been studied by microarrays (Cheung et al. 2003; Oh and Liao 2000; Phadtare and Inouye 2004; Polen et al. 2003; Richmond et al. 1999). The investigations have provided important insight into the global gene responses occurring during exposure to these single growth factors. However, still lacking in the literature are global comparative response studies. To determine the overall response, it is important to include other cellular changes, in addition to gene expression. One technique that has the potential as a screening method to study changes in the total biomolecular composition is Fourier transform infrared (FT-IR) spectroscopy (Helm et al. 1991; Helm and Naumann 1995; Moen et al. 2005; Orsini et al. 2000). The IR spectra of bacteria reflect the biochemical composition of the cell wall and membrane (phospholipids biolayer, peptidoglycan, and lipopolysaccharides) and the cellular cytoplasm (water, fatty acids, proteins, polysaccharides, and nucleic acids) (Goodacre et al. 1996; Kansiz et al. 1999; Lin et al. 2005). The spectral bands can be used to detect specific functional groups, which can be assigned to a group of biomolecules (Naumann et al. 1991). We have previously shown that FT-IR spectroscopy is a suitable technique to describe changes in the total biomolecular composition of lactic acid bacteria (Oust et al. 2004) and *Campylobacter jejuni* under nongrowth environmental conditions (Moen et al. 2005; Oust et al. 2006*a*). In particular, changes in both the lipid and the carbohydrate composition of cells can be detected using FT-IR spectroscopy.

A limiting factor for the use of DNA microarray and FT-IR spectroscopy technology is that huge amounts of data are generated, and there is a demand for highly specialized multivariate data analysis methods to extract data, identify significant responses, and compare patterns. Principal component analysis (PCA) has been shown to be well suited for analysis of the FT-IR spectra, both for identification purposes and for analysis of the biochemical information in the spectra (Næs et al. 2002; Oust et al. 2006*b*). Partial least squares regression (PLSR) has also been used successfully to detect and evaluate the covariation patterns between FT-IR spectra and DNA microarray data from *C. jejuni* (Oust et al. 2006*a*).

In this study, we have investigated the global gene expression (DNA microarray) and the total biomolecular composition (FT-IR spectroscopy) of *E. coli* growing exponentially in 10 different adverse (both natural and synthetic) conditions (sodium chloride (NaCl), ethanol (EtOH), glycerol, 2 acids (hydrochloric acid (HCl) and acetic acid (CH_3COOH)), sodium hydroxide (NaOH), heat stress (46 °C), cold stress (15 °C), ethidium bromide (EtBr) (a DNA intercalator and efflux pump inducer), and the disinfectant benzalkonium chloride (BC)). This is, to our knowledge, the first study where changes in both the global gene expression and the biomolecular composition during growth under various adverse conditions have been examined and compared. The work presents both common and unique responses to the different conditions, and illustrates the different response strategies of *E. coli* exposed to adverse conditions.

Materials and methods

Strains and growth conditions

The strain used in this study was the genome-sequenced strain *E. coli* K-12 MG1655 (Blattner et al. 1997). The effect on growth of *E. coli* under different levels of all stress factors was initially screened in a 100-well format, using the Bioscreen C online turbidity measurement instrument (MTX Lab Systems, Inc.; Vienna, Virginia) (400 μL total volume), and some levels were then selected for testing in a 40 mL volume. The stress factors, levels tested, and the final concentrations are shown in Table 1. For BC (Norwegian pharmaceutical depot), 2 concentrations were chosen, 7 and 9 μg/mL, since results from a preliminary study indicated that relatively few genes were affected by 7 μg/mL BC (data not shown). The lowest concentration of BC totally preventing growth after 24 h (the minimal inhibitory concentration) in tryptone soya broth (TSB) (Oxoid, Hampshire, UK) was 12 μg/mL (the cell concentration at inoculation was ~1×10^7 colony-forming units (CFU)/mL).

Cultures for final growth experiments were prepared by inoculating 1 colony from tryptone soya agar (TSA) (Oxoid) (overnight growth at 37 °C) to 5 mL TSB, and incubating overnight at 37 °C, with shaking (200 r/min). This culture was initially diluted 1:10 in medium and was then used to inoculate room-temperature TSB for each of the different stress factors (40 mL total volume), to a final concentration of ~1×10^7 CFU/mL (1:100 dilution of overnight culture). For the cold stress experiment, the medium was chilled to 15 °C prior to inoculation. The cultures were incubated at 37 °C, unless otherwise stated (Table 1), and shaken at 200 r/min, and samples for global gene expression and biomolecular analyses were collected when the cells had regained exponential growth at a cell density of ~1×10^8 CFU/mL. All the stress conditions, including the control, were inoculated with the same overnight culture and started at the same time point. The experiment was performed 3 times on different days and with freshly prepared solutions, resulting in 3 biological replicates.

The lag time and the generation time were calculated from a linear regression line, based on 3 points in the linear area of the $\log_{10}$ CFU/mL curve for each growth condition. The lag times (X) presented were defined as the time point

Table 1. Adverse conditions and the corresponding levels tested in Bioscreen C and in the final experiments.

Condition	Growth levels tested in Bioscreen C	Growth levels tested in 40 mL volume	Final growth level
Control	37 °C	37 °C	37 °C
CH_3COOH[a]	pH 2–7	pH 5.9	pH 5.9
HCl[a]	pH 2–7	pH 4.75	pH 4.75
NaOH[a]	pH 7–10	pH 9.6	pH 9.6
EtOH	1.5%–13.5%	5%–6%	5%
NaCl	2.5%–9.5%	4%–4.5%	4.5%
Glycerol	1%–27%	15%–18%	15%
BC	4–36 μg/mL	7–9 μg/mL	7 and 9 μg/mL
EtBr	4–450 μg/mL	100–150 μg/mL	150 μg/mL
Heat stress	—	42–48 °C	46 °C
Cold stress	—	15 °C	15 °C

Note: EtBr, ethidium bromide; BC, benzalkonium chloride.

[a]The tryptone soya broth medium was pH adjusted with the respective base or acid before inoculation with bacteria. The pH after inoculation was 6.0 ± 0.05 for CH_3COOH, 4.9 ± 0.05 for HCl, and 9.4 ± 0.05 for NaOH in cultures made for microarray and Fourier transform infrared (FT-IR) analyses.

when the linear regression line ($Y = aX + b$) equals the $\log_{10}$ CFU/mL at time 0 ($X = [\log_{10}(\text{CFU/mL at time 0}) - b]/a$). The generation time was defined as the time (Δt) needed to double the $\log_{10}$ CFU/mL, and calculated using the same linear regression lines ($\Delta t = \log_{10}2/a$). The average lag and generation times presented in Table 2 are based on 3 replicates (from the 40 mL level tests).

Biomolecular analyses (FT-IR spectroscopy): sample preparation and measurements

Two replicates (technical) from a 3–6 mL sample (depending on the optical density) were collected at the same time point as in the microarray experiments (that is, ~1×10^8 CFU/mL), centrifuged at 4 °C at 6000*g* for 1 min, and washed twice in 1 mL saline solution. After washing, the pellet was resuspended in 40 μL distilled water, and 35 μL of each suspension was transferred to an IR transparent optical crystal (ZnSe) in a multisample cuvette (Bruker Optics). The samples were dried under moderate vacuum (1×10^4 Pa), using anhydrous silica gel (Prolabo) in a dessicator to form films suitable for FT-IR analysis. The FT-IR measurements were performed with a Bio-module (Bruker Optics), specially designed for measurements of microorganisms, coupled to an Equinox 55 spectrometer (Bruker Optics). Before data analysis, the FT-IR spectra were preprocessed by calculating the second derivative prior to extended multiplicative signal correction (EMSC) (Martens and Stark 1991). The second derivative was used to resolve overlapping bands, to suppress broader underlying structures (like bands from water that is still present in the samples after drying), and to remove baseline and linear wavenumber-dependent effects. EMSC was applied to correct for variations in the effective optical path length, which may be due to sample thickness or other light-scattering effects. The interpretation of the FT-IR spectra was based on the minima of the second derivative spectra.

Table 2. Mean (±SD) lag and generation times of the *Escherichia coli* cells when exposed to the different adverse conditions.

Condition	Lag time (min)	Generation time (min)
Control	62±13	18±5
CH_3COOH (pH 5.9)	55±13	46±7
HCl (pH 4.75)	58±7	33±3
NaOH (pH 9.6)	148±4	25±1
EtOH (5%)	54±16	65±7
NaCl (4.5%)	141±6	45±8
Glycerol (15%)	47±0	61±7
BC (7 μg/mL)	133±7	23±1
BC (9 μg/mL)	384±18	18±7
EtBr (150 μg/mL)	74±12	56±6
Heat stress (46 °C)	40±4	19±1
Cold stress (15 °C)	97±78	312±19

Note: EtBr, ethidium bromide; BC, benzalkonium chloride.

FT-IR spectra of microorganisms are usually divided into 5 regions that contain information from different cell components (Naumann et al. 1991): 3000–2800 cm^{-1} for fatty acids in the bacterial cell membrane; 1700–1500 cm^{-1} for amide bands from proteins and peptides; 1500–1200 cm^{-1} for mixed regions (proteins and fatty acids); 1200–900 cm^{-1} for carbohydrates within the cell wall; and 900–700 cm^{-1} for true fingerprint region containing bands that cannot be assigned to specific functional groups. Supplement 1[5] shows a tentative assignment (based on pure compounds) of some bands frequently found in biological FT-IR spectra. In addition, assignment of various bands in the carbohydrate region has been presented by Kacuráková and Mathlouthi (1996). In this report, the interval from 3010 to 2800 cm^{-1} was used to represent the fatty acid region, since a band at

[5] Supplementary data for this article are available on the journal Web site (http://cjm.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 3960. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/eng/ibp/cisti/collection/unpublished-data.html.

~3010 cm^{-1} is assigned to the C=C–H stretch in lipids (Socrates 2001).

RNA extraction

Total RNA was extracted from a 1 mL bacterial solution (~1 × 10^8 CFU/mL), using the RNeasy Protect Bacteria Mini Prep kit (QIAGEN), as recommended by the manufacturer, including the on-column DNase treatment. The concentration and purity of the total RNA was analyzed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and the RNA 600 Nano LabChip system (Agilent Technologies).

Labeling of total RNA and DNA microarray hybridization

Total RNA (5 μg) and 0.5 μL test and reference Lucidea spike control RNA (Amersham Biosciences) were reverse transcribed, using random hexamers in the presence of aminoallyl-dUTP (2 aa–dUTP (Ambion) : 3 dTTP (Invitrogen)), as described in standard operating procedure No. M007 (The Institute for Genomic Research (TIGR) protocol). The removal of unincorporated aa–dUTP and free amines was performed according to the TIGR protocol, using the QIAGEN QIAquick PCR purification kit. After drying in a SpeedVac, the samples were stored at –20 °C. Coupling aminoallyl-labeled cDNA to Cy3/Cy5 mono reactive dyes (Amersham Biosciences) was done according to the TIGR protocol. The *E. coli* microarrays consisted of *E. coli* oligos (Operon) printed on Corning Ultra GAPS slides, as described elsewhere (Constantinidou et al. 2006; Zhang et al. 2004; Zheng et al. 2004). Microarray slides were treated with a presoak solution (Promega/Corning) before incubation in a prehybridization solution (5× standard saline citrate (SSC) buffer (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), 0.1% sodium dodecyl sulfate (SDS), and 1 mg/mL bovine serum albumin) at 42 °C for 30–60 min. After prehybridization, the slides were washed briefly in distilled water, followed by a brief wash in isopropanol. The dried Cy3- and Cy5-labeled cDNA was resuspended in 55 μL of hybridization solution (30% formamide, 5× SSC, 0.1% SDS, and 0.1 mg/mL salmon sperm DNA), denatured, and applied to the prehybridized microarray under a 25 mm × 60 mm mSeries LifterSlip cover slip (Erie Scientific Company). The slides were hybridized at 42 °C overnight (~16 h). After hybridization, the slides were washed twice in 2× SSC buffer with 0.1% SDS at 42 °C for 5 min, followed by 2 washes in 0.1× SSC buffer with 0.1% SDS for 10 min at room temperature, and 4 washes in 0.1× SSC buffer for 1 min at room temperature. The slides were dried by centrifugation (90*g* for 12 min). The reference used in the microarray experiments was pooled RNA from all the growth conditions, including the control.

Data acquisition and analysis (microarray data)

Slides were scanned with a ScanArray Express 1.0 scanner (Packard BioScience), following the manufacturer's guidelines. The fluorescent spot intensities were quantified using ImaGene version 5.6.1 software (BioDiscovery Inc.). Background subtraction and normalization (lowess) (Quackenbush 2002; Yang et al. 2002) was performed in GeneSpring version 7 (Silicon Genetics). (A lowess curve was fit to the log intensity versus log ratio plot. Twenty percent of the data was used to calculate the lowess fit at each point. The curve was used to adjust the control value for each measurement. If the control channel was <10, then the value was set to 10.) Only data from spots representing *E. coli* K-12 MG1655 genes were analyzed in our studies. All analyses were based on 3 biological replicates, with the exception of the second biological replicate from conditions HCl and NaOH. These 2 observations were removed because of bad hybridization, giving a total of 34 array hybridizations (observations). Genes not present in any of the 34 observations were filtered out, resulting in the analysis of 4279 out of 4289 MG1655 genes on the array. There were 2927 genes present in all 34 observations, resulting in 1352 genes with a missing value in 1 or more observations. Missing values (data points) were replaced using the KNNimpute procedure (Troyanskaya et al. 2001) (k = 10) on $\log_2$ transformed data. (Of the genes with missing values in 1 or more observations, 690 genes were present in >30 observations, and only 12 genes were present in just 1 observation.) To analyze explained variance and significance for the growth conditions by analysis of variance (ANOVA) modeling, we reparameterized the conditions using 11 dummy variables (0s and 1s), corresponding to all conditions except the control. A significance test for a dummy variable is then a test of difference between a specific condition and the control. To combine this model with multivariate responses from several genes, the analyses were performed according to the approach implemented in the 50–50 multivariate ANOVA (MANOVA) software (Langsrud 2002). The biological replicates were built into the model as block factors in the analyses. Significance analysis for each individual response (gene) was performed, with p value adjustment calculated according to the rotation testing principles described by Langsrud (2005). However, a modified procedure that adjusts p values according to a false discovery rate (FDR) criterion was used (Moen et al. 2005). Because of the large number of significant genes for the various growth conditions, and to avoid problems with falsely identified genes, most of the results and discussion are focused on operons containing several significant genes.

The microarray data are available through Gene Expression Omnibus (accession No. GSE11041).

Data analysis (FT-IR spectroscopy data)

The technical replicates (measurement replicates) for each sample were averaged before 50–50 MANOVA analysis and PCA. Significance analysis for the wavenumbers and corresponding absorbances in the fatty acid, protein, and carbohydrate regions of the spectra was performed, with p value adjustment calculated as described for the microarray data.

PCA

PCA is designed to find the main variation in the data matrix **X**, and to summarize this variation in a new set of variables that are linear combinations of the input variables (Næs et al. 2002). The new variables are called principal components (PCs). The first PC extracted from X contains the main variation in the data matrix **X**, whereas the second

PC contains the main variation in the data matrix **X** after extraction of the variation in the first PC, and so on. The PCs are orthogonal to each other. The result of the PCA is often presented as a score plot (as seen in Fig. 1A), and shows the main variation between the samples. PCA on the microarray and the FT-IR data were performed in Unscrambler 9.2 (Camo; Oslo, Norway). All data were analyzed weighted (1/standard deviation). The PCA score plots were based on the average of the 3 biological replicates.

Analysis of covariance in gene expression data and FT-IR spectroscopy data

The covariance of the 2 data sets was analyzed using PLSR. PLSR is related to PCA, and was designed to find the variation in data matrix **X** that is best suited to model the variation in data matrix **Y** (Næs et al. 2002). The PLSR analysis was performed in Unscrambler 9.2. The data set consisted of 3 biological replicates for all 12 conditions, except for the second biological replicate for HCl and NaOH (see analysis of microarray data), resulting in 34 samples. For the microarray data, 4279 genes were used (missing values were not imputed), whereas a selection of absorbances at 19 different minima (bands) from the second derivative spectra from the fatty acid and carbohydrate regions were used from the FT-IR data (Supplement 2).[5] Bands with increasing absorbance, compared with the control, result in negative values as a consequence of using the second derivative of the FT-IR spectra. To compare upregulated genes with upregulated absorbances, the absorbances were divided by –1. The PLSR analysis was performed with the microarray data as *X* and the selected FT-IR bands as *Y*. The data were analyzed weighted (1/standard deviation), using blockwise cross validation, where each biological replicate represents 1 block.

Results and discussion

Growth patterns

Initial high-throughput screening of a wide range of each adverse condition (Table 1) was performed using an online turbidity measurement instrument (Bioscreen C). The results from this screening (data not shown) were subsequently used to determine comparable levels for the final study (Table 1). The obtained lag and generation times from the 40 mL level tests are presented in Table 2. A considerable increase in lag time was observed for NaOH, NaCl, BC (7 and 9 μg/mL), EtBr, and cold stress (15 °C), compared with the control. All conditions, with the exception of BC (9 μg/mL), resulted in increased generation times, whereas heat stress (46 °C) had an increase of only 1 min. The response to BC differed significantly from the other conditions, and was considered to be an outlier; BC (9 μg/mL) had the longest and most variable lag time, and was related to the initial killing of ~90%–99% of the cells (as determined by plate counts). The growth of surviving cells was not due to the neutralization of BC (data not shown), and we speculate that the apparent resistance of a small subpopulation is due to adaptation over time. Adaptive resistance by repeated exposure to BC has previously been shown by other researchers (Bore et al. 2007; Braoudaki and Hilton 2005; Langsrud et al. 2003). Dynes et al. (2009) also suggested adaptation over time in situ to sub-minimum inhibitory concentrations levels of BC in *Pseudominas fluorescens* biofilms.

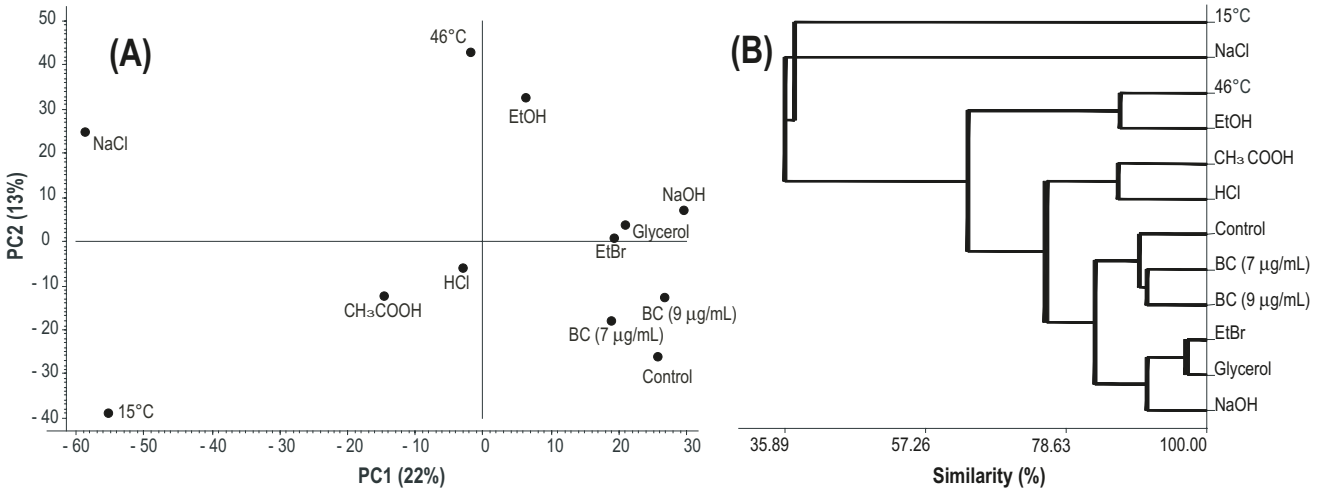
Global gene expression responses

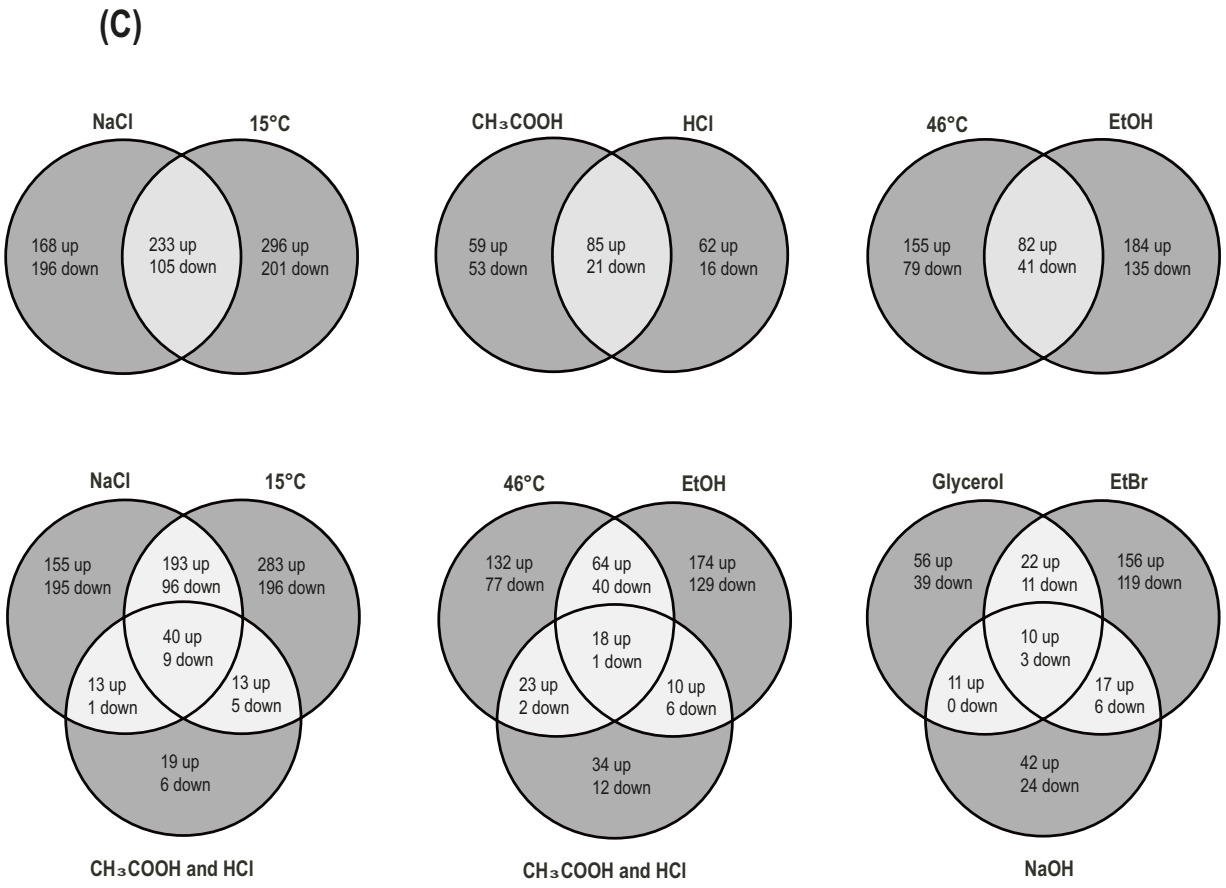
Cold stress (15 °C) and NaCl had the largest effect on gene expression data, compared with the control (Table 3 and Figs. 1A and 1B). The 2 BC concentrations did not contribute significantly to the variation in the data set (Table 3). Rotation tests (50–50 MANOVA) were performed for each condition to identify genes significantly different from the control. The number of significant genes, compared with the nonstressed control, is presented in Table 4. Gene lists with all the significant genes (FDR < 0.05) for the various conditions and $\log_2$ of fold change, compared with the control, are included in the supplementary material (Supplement 3).[5] To ensure that the number of significant genes was not a reflection of the generation time, we investigated whether there was any correlation between the number of significant genes and the length of the generation time. The analysis showed no correlation between the number of significant genes and the generation time (cold stress was considered to be an outlier, since low temperature results in slower growth than the other conditions, and was consequently removed from this analysis). Compared with the other conditions, exposure to BC resulted in few changes in gene expression (Table 4), in addition to the observed diverging growth response stated above. Therefore, the following analyses and interpretations do not include BC unless otherwise stated.

In total, approximately 40% of all genes (1682/4279) significantly changed expression, compared with the nonstressed control, of which 923 genes were upregulated, 645 were downregulated, and 114 were both up- and down-regulated. These genes are referred to hereafter as stress-related genes, although one cannot rule out the possibility that some genes may be growth related. Only 3 genes (*b2670* (or *ygaW*), *rmf*, and *ycdW* (or *ghrA*)) were significantly different (upregulated) from the control in all conditions. Gene *b2670* (or *ygaW*) encodes a protein of unknown function and belongs to a family of several hypothetical bacterial proteins. Gene *ycdW* (or *ghrA*) encodes a glyoxylate/hydroxypyruvate reductase (Nunez et al. 2001). The *rmf* gene encodes a ribosome-modulation factor known to stabilize ribosomes in stationary phase (Selinger et al. 2000; Wada et al. 1995). It is known that *rmf* protects *E. coli* under heat stress in stationary phase (Niven 2004), and it has also been reported that *rmf* is expressed in the exponential phase under slow-growth conditions (Yamagishi et al. 1993). Another stationary-phase-induced ribosome-associated protein that was significantly upregulated in most growth conditions (except BC and glycerol) was *rpsV*. There is no precise function assigned to *rpsV*, but it has been suggested that it may play a role similar to that of *rmf* (Lacour and Landini 2004; Selinger et al. 2000). These ribosome-associated proteins should be examined in more detail to determine their role in stabilizing the ribosomes during various adverse conditions.

The few common stress-related genes was surprising, considering the high total number of significant genes, suggesting that adverse conditions trigger a wide range of responses. This illustrates the diverse response potential of

Fig. 1. (A) Principal component (PC) score plot, (B) dendogram based on the principle component analysis (PCA) coordinates from the PCA of the gene expression data, and (C) Venn diagrams of shared significant genes between selected adverse conditions. The input data for A and B were the average of the 3 biological replicates, normalized $\log_2$ of ratio (pooled reference), without missing data imputed. The explained variances are shown in the plot.





E. coli and proposes a specialized ability to adapt to different environments. This aspect was analyzed further by identifying unique up- and down-regulated genes for each condition (Supplement 4).[5] The list of significant genes for each individual condition was compared with a pooled gene list of significant genes for all other conditions. Table 4 shows the number of unique genes and the percentage of unique genes compared with the total number of significant genes. NaCl, heat stress (46 °C), EtBr, EtOH, and cold stress (15 °C) had a high number of unique genes, whereas the acids (HCl and CH_3COOH), glycerol, and NaOH had few unique genes. This is in accordance with the idea that

Table 3. Explained variance and significance (by 50–50 multivariate analysis of variance) of the gene expression data and the Fourier transform infrared (FT-IR) spectroscopy data.

	Explained variance			
Condition	Gene expression	Fatty acid region	Protein region	Carbohydrate region
CH_3COOH	0.043***	0.071***	0.167***	0.121***
HCl	0.033***	0.055***	0.074***	0.082***
NaOH	0.034***	0.048***	0.109***	0.054***
EtOH	0.052***	0.087***	0.336***	0.268***
NaCl	0.070***	0.027***	0.120***	0.101***
Glycerol	0.037***	0.029***	0.107***	0.075***
BC (7 μg/mL)	0.016*	0.005*	0.046***	0.020***
BC (9 μg/mL)	0.019*	0.012***	0.057***	0.028***
EtBr	0.048***	0.071***	0.080***	0.020**
Heat stress (46 °C)	0.050***	0.056***	0.147***	0.096***
Cold stress (15 °C)	0.072***	0.084***	0.125***	0.145***

Note: Fatty acid region, 3010–2800 cm^{-1}; protein region, 1700–1500 cm^{-1}; and carbohydrate region, 1200–900 cm^{-1}. EtBr, ethidium bromide; BC, benzalkonium chloride. Significance is designated as follows: *, $p > 0.1$; **, $p = 0.1$–0.05; and ***, $p < 0.05$.

Table 4. Number of significant genes (5% false discovery rate (FDR)) compared with the control, number upregulated and downregulated, and number and percentage of unique genes for the specific conditions.

		No. of significant genes			
Condition	Total No. of significant genes (5% FDR)	Upregulated	Downregulated	Unique	% of unique genes from total No. of significant genes
CH_3COOH	218	144	74	30	13.8
HCl	184	147	37	7	3.8
NaOH	113	80	33	19	16.8
EtOH	442	266	176	110	24.9
NaCl	702	401	301	221	31.5
Glycerol	152	99	53	20	13.2
BC (7 and 9 μg/mL)	3	3	0	0	—
EtBr	344	205	139	107	32.0
Heat stress (46 °C)	357	237	120	97	27.2
Cold stress (15 °C)	835	529	306	292	35.0

Note: EtBr, ethidium bromide; BC, benzalkonium chloride.

some stress factors, such as acid, impose a general stress response, whereas heat stress, NaCl, and H_2O_2 induce more specific stress responses (Foster and Moreno 1999).

Specific and overlapping gene expression responses

The *rpoS* gene (σ^S), which acts as the master regulator of stationary-phase response and general stress response (Storz and Hengge-Aronis 2000), was upregulated by acid stress (CH_3COOH and HCl), NaOH, EtOH, NaCl, heat stress (46 °C), and cold stress (15 °C). The *rpoH* gene was, as expected, upregulated by heat stress (46 °C), and did not change significantly in any of the other conditions. This gene encodes the σ^H factor and is required for the transcription of heat shock genes (Storz and Hengge-Aronis 2000). The σ^H regulon consists of at least 30 heat shock proteins (Storz and Hengge-Aronis 2000), many of which were upregulated by heat stress in this study (see the following). The *rpoE* gene, encoding the σ^E, was upregulated by cold stress (15 °C), and was not significantly changed by any of the other conditions. σ^E is essential for the maintenance of cell envelope integrity in gram-negative bacteria (De Las Penas et al. 1997). The σ^E regulon includes a number of genes, among which are those encoding chaperones that aid outer membrane folding and the biosynthetic enzymes that are involved in phospholipid, fatty acid, lipopolysaccharide, and membrane-derived oligosaccharide synthesis and transport (Hayden and Ades 2008; Rowley et al. 2006), some of which were affected by cold stress in this study (see the following). The *rpoE* gene is cotranscribed with *rseA*, encoding an anti-sigma factor, which was also upregulated by cold stress (15 °C). Both genes have previously been reported to change during cold shock in *E. coli* (Polissi et al. 2003). The *rpoN* gene was upregulated by NaCl and cold stress (15 °C), and the housekeeping sigma factor, *rpoD* (member of the σ^E regulon), was downregulated by cold stress (15 °C). The latter effect was probably related to the decreased growth rate, compared with the control.

CH_3COOH and HCl

The 2 acids (CH_3COOH and HCl) clustered close to each other (Figs. 1A and 1B) and had many genes significantly different from the control in common (85 upregulated and 21 downregulated genes) (Fig. 1C and Table 5). Among the common upregulated genes were the glutamic acid decarboxylase genes (*gadAB*, *xasA* (or *gadC*), and *yhiEWX* (or *gadEWX*)). The *gad* system enables survival in extreme

acid (Tramonti et al. 2002). The acid chaperone genes (*hdeAB*) were also upregulated, and *hdeD* was additionally upregulated by CH_3COOH. The osmotic adaptation genes (*osmBY*) were also common upregulated genes. Many genes involved in energy metabolism were upregulated by acid, for example, the anaerobic respiration genes (*narGIK*). In addition, the reductive carboxylate cycle genes (*acnAB*), the citric acid cycle genes (*fumAC* and *sucABC*), and the oxidative phosphorylation genes (*nuoEFN*) were upregulated by HCl. The oxidative phosphorylation genes (*appBCY*) were upregulated by CH_3COOH, and the *appBC* genes were also unique for CH_3COOH. The cyclopropane fatty acid gene (*cfa*) was upregulated by CH_3COOH and HCl and, to some extent, by NaCl, EtOH, and heat stress. Increasing the amount of cyclopropane fatty acids in the outer membrane has been shown to reduce the permeability of protons (Wang and Cronan 1994), and is a major factor in acid resistance in *E. coli* (Chang and Cronan 1999). *cfa* mutants have also been reported to be more sensitive to EtOH than the parental strain (Grogan and Cronan 1997), and an increase in cyclopropane fatty acids has been reported to be stimulated by the addition of NaCl and heat shock (Marr and Ingraham 1962; McGarrity and Armstrong 1981).

Genes significantly different from the control in both acids (CH_3COOH and HCl) were compared with NaCl and cold stress (15 °C), as well as heat stress (46 °C) and EtOH (Fig. 1C and Table 5). The 2 acids had 40 upregulated and 9 downregulated genes in common with NaCl and cold stress (15 °C). Among the common upregulated genes were the glutamic acid decarboxylase genes (*gadAB* and *yhiWX* (or *gadWX*)) and the osmotic adaptation gene (*osmY*). The 2 acids had 18 upregulated genes and 1 downregulated gene in common with heat stress (46 °C) and EtOH. Among the upregulated genes were *yhiWX* (or *gadWX*), the cyclopropane fatty acid gene (*cfa*), the multistress response gene (*osmB*), and the multiple antibiotic resistance gene (*marB*); the down-regulated gene was *fadL* (encoding an outer membrane protein required for long-chain fatty acid transport).

NaOH

NaOH clustered close to glycerol and EtBr (Figs. 1A and 1B), with 10 upregulated and 3 downregulated genes significantly different from the control in common (Fig. 1C). The *malPZ* genes (maltose metabolism) and the putative gene *ynaF* (sequence similarities to the universal stress protein *uspF* (Saveanu et al. 2002)) were upregulated in all 3 conditions. NaOH caused upregulation of the glycine cleavage genes (*gcvHPT*), genes involved in the transport and degradation of maltose (*malEPZ*), and the phage shock proteins (*pspABCDE*). The phage shock protein operon has previously been reported to be expressed in response to heat stress, osmotic shock, filamentous phage infection, and exposure to EtOH (Brissette et al. 1990; Kobayashi et al. 1998). Bacteria lacking the *pspABC* genes have also been shown to exhibit a substantial decrease in the ability to survive in stationary phase under alkaline conditions (pH 9) (Weiner and Model 1994). The thiamin biosynthesis genes (*thiCDEFGHM*) were upregulated, and part of this operon (*thiDEFGHM*), in addition to *tbpA* (thiamin binding protein), was unique for NaOH and did not change significantly in the other conditions. The *yceI* gene (hypothetical protein) was upregulated by NaOH and unique to NaOH. This gene encodes a periplasmic protein previously shown to be induced during alkaline stress (Maurer et al. 2005; Stancik et al. 2002). Alkaline stress has not been studied to the same extent as acidic stress (Saito and Kobayashi 2003), and there are few reports of genes involved in alkaline stress in *E. coli*. The upregulation of the thiamin biosynthesis genes were unique to NaOH and have, to our knowledge, not previously been reported.

EtOH

EtOH clustered close to heat stress (46 °C) (Figs. 1A and 1B), with 82 up- and 41 down-regulated genes significantly different from the control in common (Fig. 1C and Table 5). Among these, the heat shock genes (*clpB*, *dnaK*, *htrA*, and *ibpA*), the inducible heat shock gene *lysU*, and the multiple antibiotic resistance protein genes (*marAB*) were upregulated, whereas the fatty acid genes (*fadDL*), the lipopolysaccharide core biosynthesis gene (*rfaC*), and the sugar–nucleotide biosynthesis genes (*rffGH*) were downregulated. Additionally, EtOH induced upregulation of the purine and pyrimidine metabolism genes (*deoABCD* and *nrdDEHI*), the galactose and phosphotransferase genes (*gatABCDRYZ*), the glucitol genes (*srlABDE*), the multiple antibiotic resistance gene (*marR*), genes involved in mannose metabolism and transport (*manAXYZ*), genes involved in transport and metabolism of maltose (*malEFMPTZ*), and the phage shock genes (*pspABCD*). The oxidative phosphorylation genes (*cyoABCDE* (unique to EtOH)), the fatty acid and phosphatidic acid biosynthesis genes (*fabBDH*), the lipopolysaccharide core biosynthesis genes (*rfaCPZ*), the sugar–nucleotide biosynthesis genes (*rfbACDX*), and the enterobacterial common antigen genes (*wecBCDG* (or *rffEDCM*)) were downregulated.

NaCl

NaCl was separated from all other conditions (Figs. 1A and 1B), but was close to cold stress (15 °C) on PC1. The conditions shared 233 up- and 105 down-regulated genes significantly different from the control (Fig. 1C and Table 5). Genes involved in osmoprotection (*osmCEY*, *otsAB*, *yehZYXW* (Checroun and Gutierrez 2004), *betIT*, *proP*, *proV*), genes involved in modification of polysaccharides (*glgABPX*), the citric acid cycle genes (*sdhABCD* and *sucACD*), and the glutamic acid decarboxylase genes (*gadAB* and *yhiWX* (or *gadWX*)) were among the common upregulated genes. Common downregulated genes included the ferric citrate transport genes (*fecACER*), the fimbriae genes (*fimFI*), and the flagella genes (*flhE* and *fliJN*). In addition to the common genes were the osmotic adaptation gene (*osmB*), the osmoprotectant transport genes (*proWX* (unique for NaCl)), and the osmoprotectant synthesis gene (*betA*) upregulated by NaCl. Among the unique genes upregulated by NaCl were the colanic acid genes (*wcaABCFGIJKM*, *wza*, and *cpsBG* (renamed *manCB*)). The positive regulatory gene for capsule (colanic acid; *rcsA*) was also upregulated. It has been suggested that colanic acid is important for the survival of *E. coli* outside the host (Ophir and Gutnick 1994), and it has been reported to protect *E. coli* O157:H7 from osmotic stress (Chen et al. 2004). The upregulated genes *proVWX*, *otsA*, and *b1481* (also upregulated in

Table 5. Summary of overlapping molecular functions between selected conditions.

	Condition				
Molecular function	CH_3COOH and HCl	CH_3COOH and HCl, NaCl and 15 °C	CH_3COOH and HCl, EtOH and 46 °C	EtOH and 46 °C	NaCl and 15 °C
Carbohydrate metabolism					
Glutamate decarboxylase	***gadABCDEWX***	***gadABWX***	***gadWX***	***gadWX***	***gadABWX***
Lipopolysaccharide core biosynthesis				*rfaC*	*rfaH*
Modification of polysaccharides					***glgABPX***
Chaperones					
Acid chaperones	***hdeAB***				
Heat chaperones				***clpB, dnaK, htrA, ibpA, lysU***	
Energy metabolism					
Anaerobic respiration	***narGIK***				***narZ,*** *narP*
Oxidative phorphorylation	***appY***				
Reductive carboxylate cycle	***acnA***	***acnA***			***acnA***
TCA	***fumA, sucB***				***fumC, sdhABCD, sucACD***
Fatty acid					
Cyclopropane fatty acid	***cfa***		***cfa***	***cfa***	
Fatty acid transport	*fadL*		*fadL*	fadDL	
Osmotic adaptation	***osmBY***	***osmY***	***osmB***	***osmB***	***osmCEY, otsAB, yehZYXW, betIT, proPV,*** *proB*
Resistance					
Multiple antibiotic resistance	***marB***		***marB***	***marAB***	
Surface structure					
Capsule regulatory genes					***rcsA***
Fimbriae					*fimFI*
Flagella					*flhE, fliJN*

Note: TCA, tricarboxylic acid cycle. Upregulated genes are indicated in boldface and italic type. Downregulated genes ae indicated in italic type.

this study; see Supplement 3)[5] were among the top 5 upregulated genes presented in an osmotic stress microarray study by Cheung et al. (2003). In addition to the common downregulated genes (with cold stress (15 °C)) were the fimbrial genes (*fimCD*) and the flagella genes (*flgM*, *flhB*, and *fliL*) downregulated by NaCl.

Glycerol

Glycerol induced genes involved in the galactose metabolism (*galMPT*), the glycerol phosphate genes (*glpKQT*), and genes involved in the transport and metabolism of maltose (*malEFMPQTZ*). Genes involved in transport of small molecules (*glnHPQ* and *potABCD*) were downregulated.

BC (7–9 μg/mL)

There were no genes with a FDR <0.05 in BC (7 μg/mL), and only 1 gene (*ykfE* (or *ivy*)) in BC (9 μg/mL). The 2 BC concentrations were therefore analyzed as 1 condition, resulting in 3 genes (*ykfE*, *osmB,* and *b1171*) significantly different from the control. Gene *ykfE* (or *ivy*) encodes an inhibitor of C-lysozyme (Deckers et al. 2004; Monchois et al. 2001), *osmB* encodes a multistress response gene, and gene *b1171* has no known function. The low number of significant genes was surprising, considering the huge effect BC has on the cells in the form of cell death and morphological changes (data not shown). Reinoculation studies (log growth cells grown with and without BC reinoculated to fresh medium with and without BC) resulted in a lag time for cells pregrown in BC similar to that of nonstressed cells reinoculated to medium without BC (data not shown). We interpret these results to mean that the low number of significant genes was not due to neutralization of BC in the medium during growth, and that the surviving subpopulation may have adapted to BC during the long lag time.

EtBr

EtBr induced many genes involved in DNA repair (*dinFGIP*, *lexA*, *recAN*, and *uvrAB*). Many of these genes (*dinFI*, *lexA*, *recA*, and *uvrA*) were also unique for EtBr, and did not change significantly in the other conditions. Interesting genes unique (upregulated) to EtBr were the SOS-inducible genes *yafNOP*. *yafNOP* genes have previously been shown to be SOS-inducible and part of the 4 gene operon, *dinB-yafNOP* (Courcelle et al. 2001; McKenzie et al. 2003). McKenzie et al. (2003) have suggested that *yafNOP* gene products contribute to spontaneous mutation in growing cells. EtBr is often used as a model substrate to induce efflux pumps in gram-negative bacteria (Li and Nikaido

2004), but genes involved in known efflux pumps were not induced in this study. Only 1 of the many putative drug transporter genes analyzed by Nishino and Yamaguchi (2001), gene *yieO*, was significantly upregulated by EtBr. Among the downregulated genes were the *oppCDF* genes (similar to *Salmonella* oligopeptide ABC transport system) and the spermidine/putrescine transport genes (*potABCD*).

Heat stress (46 °C)

Heat stress (46 °C) induced, as expected, many of the known heat shock proteins and chaperones (*clpB*, *dnaK* (Hsp70), *htpGX*, *htrA*, *ibpA*, and *lysU*). In addition, the σ^H factor (*rpoH*) was upregulated and unique for heat stress. Another known heat shock gene that was upregulated was the *yedU* gene (Hsp31) (Richmond et al. 1999; Sastry et al. 2002). One cold shock protein gene (*cspD*) was also upregulated. The surface polysaccharide/lipopolysaccharide genes (*kdtAB*) (unique to heat stress (46 °C)), the multiple antibiotic resistance protein genes (*marAB*), and the lipoprotein modification genes (*nlpCD*) were also among the upregulated genes. The cold shock protein genes (*cspAEG*) (*cspEG* unique (downregulated) to heat stress (46 °C)), the fatty acid genes (*fadDLR*), the flagella genes (*flgBCJ*) (*flgBC* unique to heat stress (46 °C)), the oligopeptide transport genes (*oppBCF*), the lipopolysaccharide core biosynthesis genes (*rfaCQ*), and the sugar–nucleotide biosynthesis genes (*rffGH*) were among the downregulated genes.

Cold stress (15 °C)

Cold stress (15 °C) was the condition that resulted in the highest number of genes significantly different from the control (Table 4). Among those upregulated were, as expected, the cold shock protein genes (*cspBFGI* (*cspBI* unique to cold stress (15 °C))). The *cspA* gene did not change significantly, and this can be explained by the fact that samples for analysis were taken after a prolonged period at a low temperature (that is, when cells had regained exponential growth). CspA mRNA is highly stable throughout the cold shock period, but the stability is lost after a short time, and the mRNA is then degraded (Goldenberg et al. 1996). As mentioned before, the *rpoE* (encoding σ^E) and the cotranscribed gene *rseA* were upregulated. Other upregulated members of the σ^E regulon were the *ddg* (*lpxP*) and *psd* genes, involved in lipid A expression/biosynthesis and phospholipid biosynthesis, respectively. The capsule regulatory genes (*rcsABC*) were also upregulated (*rcsBC* was unique for cold growth). As also stated in the NaCl section, genes involved in osmoprotection, polysaccharide modification, the citric acid cycle, and the glutamic acid decarboxylase genes were upregulated. Phadtare and Inouye (2004) reported that some of these genes (e.g., *cspBGI* and *otsAB*) are upregulated in a genome-wide transcriptional analysis of the cold shock response. The protein transport genes (*ccmABH*) (unique to cold stress (15 °C)), the electron transport genes (*cydABD*), the citrate-dependent iron transport genes (*fecACER*), the fimbriae genes (*fimACFI*), the flagella genes (*flhE* and *fliGJN*), and the sigma factor *rpoD* (unique to cold stress (15 °C)) were among the downregulated genes. *fecACE* genes were also reported to be downregulated in response to cold shock by Phadtare and Inouye (2004).

Summary of overlapping gene expression responses

A number of the adverse conditions had many overlapping molecular functions, some of which are summarized in Table 5. However, only 3 genes significantly changed expression, compared with the control, in all conditions (*b2670* (or *ygaW*), *rmf*, and *ycdW* (or *ghrA*)). Among these, the ribosomal modification factor (*rmf*), known to stabilize ribosomes in stationary phase, is of special interest. We speculate that this ribosomal modification factor plays an important role during a number of adverse conditions, not only during stationary phase. The high number of total genes significantly different from the control and the few common genes reflects the ability of *E. coli* to adapt to different adverse conditions, and stands in contrast to the few genes needed for maintaining basic growth under optimal growth conditions (Galperin 2006; Glass et al. 2006; Hashimoto et al. 2005; Posfai et al. 2006). Among the genes that were common between many of the stress conditions (Table 5) were genes involved in the *gad* system. This system is known to be important for acid stress (Tramonti et al. 2002), but we have shown that part of this system is also upregulated by NaCl, cold stress, EtOH, and heat stress. Many genes involved in energy metabolism were also common between the 2 acids (CH_3COOH and HCl) and between NaCl and cold stress (15 °C). NaCl and cold stress (15 °C) also shared many osmotic adaptation genes.

Biomolecular responses (FT-IR)

Table 3 shows the different growth conditions and their corresponding explained variance and significance of the fatty acid region (3010–2800 cm^{-1}), the protein region (1700–1500 cm^{-1}), and the carbohydrate region (1200–900 cm^{-1}). Supplement 5[5] presents a selection of wavenumbers with an FDR <0.05, and the corresponding *t* values (by 50–50 MANOVA). EtOH was the condition with the largest effect on all 3 regions of the spectra, compared with the control, with 8.7% explained variance in the fatty acid region, 33.6% in the protein region, and 26.8% in the carbohydrate region (Table 3). EtOH has previously been reported to affect protein folding and (or) denaturation (Storz and Hengge-Aronis 2000), and is known to have a large influence on the fatty acid composition because it induces synthesis of lipids containing elevated amounts of unsaturated fatty acids (Chiou et al. 2004; Ingram 1976). EtOH was, together with cold stress (15 °C), the only condition with a significant increase in band (~3006 cm^{-1}; Supplement 2),[5] which is assigned to the C=C–H stretch present in unsaturated fatty acids. The same band is decreased by exposure to both acids and heat stress. Correspondingly, the microarray analysis showed that the *cfa* gene was increased by acid, NaCl, EtOH, and heat stress. The decrease in unsaturated fatty acids by acid and heat stress could be explained by the fact that cyclopropane fatty acid syntase inserts a methylene group across the double bond, resulting in loss of cis fatty acids (Grogan and Cronan 1997). This renders the lipid biolayers more rigid, thereby decreasing the membrane fluidity (Loffhagen et al. 2007). Interestingly, EtOH had both an increase in unsaturated fatty acids and *cfa* upregulation. We currently do not have an explanation for this observation, but both unsaturated fatty acids and cyclopropane fatty acids have been reported to increase with EtOH exposure (Chiou

et al. 2004; Grogan and Cronan 1997; Ingram 1976; Ku et al. 2007). Loffhagen et al. (2007) also measured membrane fluidity as a function of temperature, using the position of the maximum of the symmetric vibration band of the CH_2 group (~2850 cm^{-1}). They found that the position of the CH_2 stretching band of cells containing trans fatty acids were at lower wavenumbers than those without trans fatty acids, indicating reduced fluidity. The FT-IR data from our heat and cold experiments indicate a similar increase in fluidity from heat stress to control to cold stress. This was interpreted from a shift around bands ~2960 and ~2851 cm^{-1} between cold stress (15 °C), heat stress (46 °C), and the control; that is, the maximum of the CH_2 stretching was shifted from 2851 cm^{-1} in the control to 2852 cm^{-1} in cold stress (15 °C). Correspondingly, the maximum of the CH_2 stretching is shifted from 2922 cm^{-1} in the control, to 2921 cm^{-1} in heat stress (46 °C) and 2923 cm^{-1} in cold stress (15 °C). The FT-IR data also gave indications of changes in fatty acid chain lengths. When the CH_3/CH_2 ratio was calculated, there was a significant increase in both both NaOH and EtOH, compared with the control. We interpret this to indicate shorter chain lengths than in the control. The decrease in fatty acid chain length observed for EtOH could be related to the downregulation of *fabBHD* and *fadDL* (involved in fatty acid elongation and transport).

Figure 2 shows PCA score plots and dendograms of the different regions of the spectra. In the fatty acid region, cold stress (15 °C) was separated from the other conditions (Figs. 2A and 2B) and was, together with EtOH, the condition with the largest effect on the fatty acid composition (Table 3). The 2 acids and NaOH were differentiated by PC1 and PC2 (Fig. 2A), and induced opposite effects on bands assigned to the C–H stretch of CH_2 (~2921 and ~2851 cm^{-1}), increasing by acid and decreasing by NaOH. Heat stress (46 °C) and cold stress (15 °C) conditions were differentiated by PC2, and this PC was mainly influenced by band ~3006 cm^{-1} (C=C–H stretch), reflecting the difference in unsaturated fatty acids.

In the protein region, the control was differentiated from the growth conditions by PC1 (Figs. 2C and 2D), with EtOH farthest off. The separation on PC1 was influenced by an increase in several bands during stress (assigned to α-helical structures of amide I and amide II), indicating that the stress conditions induced major global changes in the protein composition, secondary structure, and folding.

In the carbohydrate region, EtOH and cold stress (15 °C) clustered separately from each other and from the remaining conditions (Figs. 2E and 2F). EtOH was separated from the other conditions on PC1, whereas cold stress (15 °C) was separated from the other conditions on PC2 (Fig. 2E). PC1 was influenced by a decrease in several bands during stress, compared with the control, whereas PC2 was mainly influenced by bands ~1154 and ~1026 cm^{-1}. Bands ~1154 and ~1026 cm^{-1} increased significantly, compared with the control, during most conditions, with the largest increase being in cold stress (15 °C) (Supplement 2);[5] these bands have previously been associated with survival of *C. jejuni* under nongrowth conditions (Moen et al. 2005; Oust et al. 2006*a*). These bands may be related to changes in the carbohydrate composition of the outer membrane during cold stress, possibly related to the cold induced upregulation of genes involved in modification of polysaccharides and capsule regulatory genes (see specific gene expression responses).

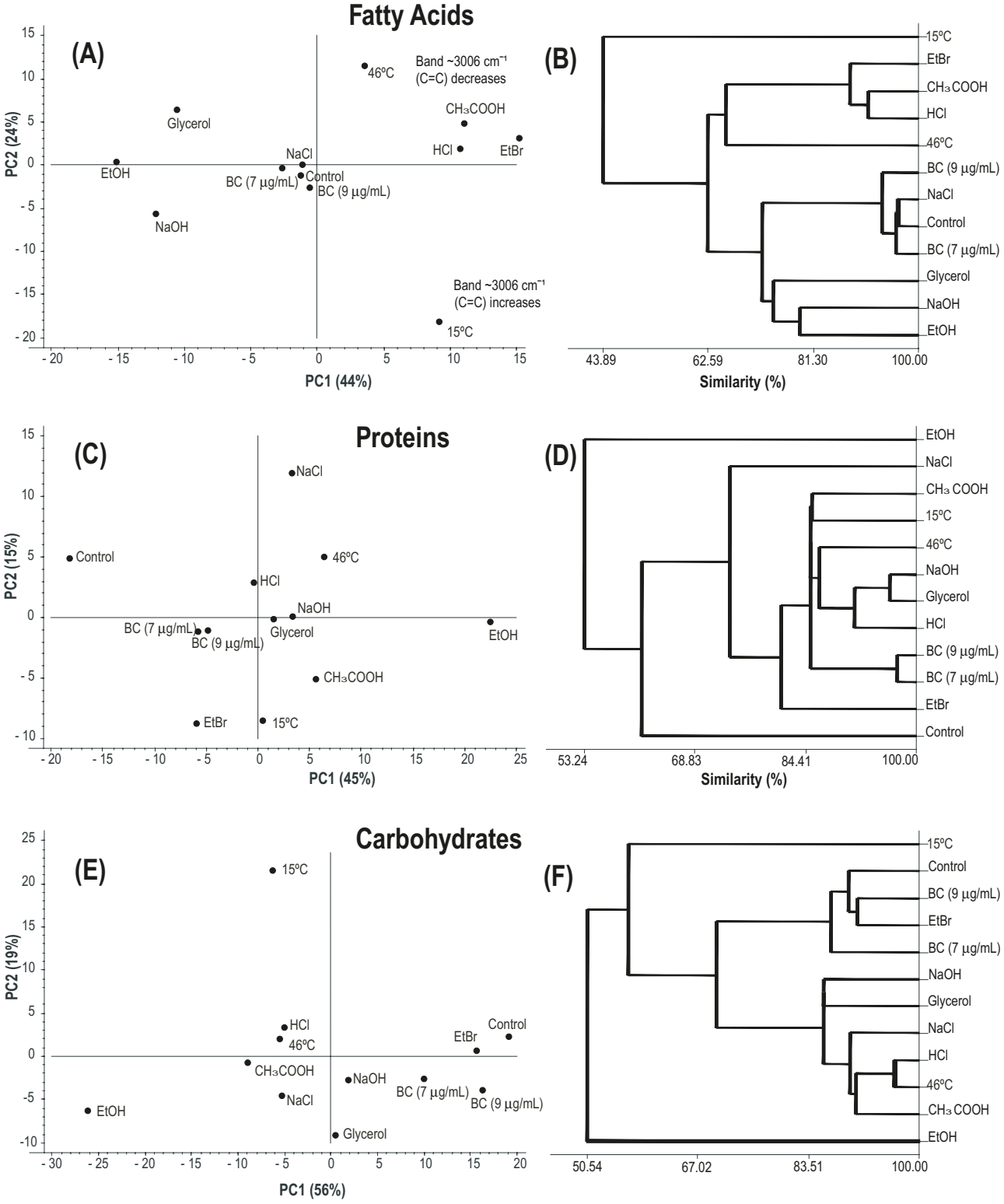
In addition to the fatty acid, protein, and carbohydrate regions, other regions of the spectra were analyzed to identify interesting bands and corresponding significant change, compared with the control. This analysis identified bands ~1742 and ~1400 cm^{-1}. Band ~1742 cm^{-1} is assigned the C=O stretch of esters, and band ~1400 cm^{-1} is assigned the C=O stretch of COO^-. Band ~1742 cm^{-1} was increased by acid, EtBr, and cold stress (15 °C), compared with the control, whereas band ~1400 cm^{-1} was only significantly changed (increased) by NaOH and cold stress (15 °C) (Supplement 2).[5]

Regression analysis between the gene expression and the FT-IR data

To detect possible covariance structures in the data set, the gene expression data (all genes) (*X*) and a selection of 19 bands (*Y*) in the FT-IR spectra (the same bands that are represented in Supplement 2)[5] were linked together by running a PLSR. The PLSR analysis showed that most of the variation seen in the 2 first partial least squares components was due to changes in EtOH and cold stress (15 °C) conditions, compared with the control (data not shown). The PLSR analysis revealed a relatively low correlation between the gene expression data and the biomolecular data. However, an interesting result from the PLSR analysis was the high correlation between the carbohydrate-associated bands ~1154 and ~1026 cm^{-1} and a selection of genes induced by cold stress (15 °C). Among these genes were many with unknown function, genes *glgAP* (glycogen syntase and phosphorylase), and the positive regulatory gene for capsule (colanic acid) (*rcsA*). This correlation was substantiated by plotting of the raw data (that is, plotting bands ~1154 and 1026 cm^{-1} against the *glgAP* and *rcsA* genes, separately). This is in accordance with previous observations, suggesting that these bands are related to changes in the carbohydrate composition of the outer membrane (Moen et al. 2005; Oust et al. 2006*a*). Another interesting positive correlation was observed between band ~1742 cm^{-1} (assigned the C=O stretch of esters) and the *ddg* (*lpxP*) gene induced by cold stress (15 °C). The *ddg* (*lpxP*) gene encodes lipid A expression/biosynthesis or, more specifically, palmitoleoyl transferase. Palmitoleate is not present in lipid A isolated from *E. coli* cells grown above 30 °C, but has been shown to comprise ~11% of the fatty acid acyl chains of lipid A in cells grown at 12 °C (Carty et al. 1999).

In this study, we have, for the first time, analyzed the effect of several different adverse conditions in the same study, using 2 explorative approaches (microarray and FT-IR spectroscopy). The various conditions triggered a large variation of responses, but results from the microarray analysis identified few common genes between the responses. This illustrates the vast adaptation potential of *E. coli*. Except for the covariance between 2 carbohydrate-associated bands and genes related to glycogen metabolism and capsule, the ester-associated band and the lipid A expression/biosyntesis gene, the PCA and regression analysis revealed that the gene expression and the biomolecular responses are not well correlated in stressed populations of *E. coli* K12. This underlines the importance of multiple strategies in the quest to understand the effect on the whole cell.

Fig. 2. (A, C, and E) Principal component (PC) score plots and (B, D, and F) dendograms based on the principle component analysis (PCA) coordinates from the PCA of the FT-IR spectroscopy data. The analyses were based on the average of the 3 biological replicates. The fatty acid region (3010–2800 cm^{-1}) is shown in A and B, the protein region (1700–1500 cm^{-1}) is shown in C and D, and the carbohydrate region (1200–900 cm^{-1}) is shown in E and F. The explained variances are shown in the plot.



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